The Roles of Serine−55 in *E. coli* Ornithine Transcarbamylase (Cloning and Expression of *E. coli argI* gene mutated at Serine−55)

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ABSTRACT

E. coli ornithine transcarbamylase is the enzyme which catalyzes the synthesis of citrulline from ornithine and carbamyl phosphate in the arginine biosynthetic pathway. The enzyme has the stretch of conserved amino acid sequences, -Ser-Thr-Arg-Thr-, which many phosphate binding proteins have. The stretch is assumed to contribute the binding of carbamyl phosphate to the enzyme, which initiated the induced-fit type of enzymic conformational change. The purpose of the project was to study the roles in catalytic action of Ser-55 in *E. coli* ornithine transcarbamylase. The serine residue was mutated to a cysteine, an alanine and a glycine. They were different in the size and the charge each other. DNA polymerase chain reaction was used in the site-directed mutagenesis of *arg* I gene at Ser-55. The results of gel electrophoresis suggested that mutant *arg* I genes substituted Ser-55 to cysteine, alanine and glycine were successfully constructed on the expression vector, pKK223-3. Wild type and Cys-55 mutant enzymes were expressed in *E. coli* TB2 strain which had a partially deleted a wild-type *arg* I gene. The result of SDS-PAGE showed the wild-type enzyme is highly purified. The purification of Cys-55 mutant enzyme has been undergoing. DNA sequencing of mutant genes, and the expressions and characterizations of other mutant enzymes will be performed.

[Supported by grants from MOE]

INTRODUCTION

Ornithine transcarbamylase (2.1.3.3) catalyzes the condensation reaction of ornithine and carbamyl phosphate to produce citrulline in the arginine biosynthetic pathway. The enzyme is composed of three identical subunits. Its molecular weight is about 110 kilodalton. *E. coli* ornithine transcarbamylase normally observes Michaelis-Menten kinetics for both substrates, ornithine and carbamyl phosphate. The regulation of enzymic activity is mediated by Zn^{2+} ion. Zn^{2+} regulates the enzyme in two routes on the basis of the incubation patterns. The metal behaves as the classical reversible allosteric inhibitor to the binary complex of enzyme and carbamyl phosphate, while it acts as the tight binding irreversible inhibitor to the enzyme alone. This irreversible inhibition is incubation-time dependent. One of the enzymic mode of action behaved by ornithine transcarbamylase has been understood. The enzyme undergoes the conformational change as the first substrate carbamyl phosphate binds, which results opening the active site pocket for second substrate ornithine to bind.

Several amino acids such as arginine, lysine, cystein were proposed as critical ligands for enzyme functions. *E. coli* Arginine-57 plays an important role in carbamyl phosphate-induced enzyme isomerization. Cysteine-273 is a ligand of ornithine and Zn^{2+}. Except these, the overall pictures for the catalysis and ligand binding at the active site have not been well elucidated. Since Ser-Thr-Arg-Thr is the conserved amino acid sequences of phosphate binding site for many proteins, and *E. coli* ornithine transcarbamylase has a same stretch of amino acid sequences, this study will focus on the structural and functional roles of Serine-55 which is one of amino acids on this stretch. Cloning of three mutant genes for Serine-55 were performed to characterize the kinetic behaviors of mutane enzymes.

MATERIALS AND METHODS

Materials

Oligodeoxynucleotides and Taq DNA polymerase for polymerase chain reaction (PCR) were purchased from Korea Biotech. Restriction enzymes and deoxynucleotides were obtained from

BM Korea, and prokaryotic expression vector, pKK223-3 was purchased from Pharmacia. Matrex Blue Gel A for affninity chromatography was obtained from Amicon (USA). Other chemicals for DNA and protein works were purchased from SIGMA (USA).

Methods

Site-directed mutagenesis : PCR methods were applied to synthesize the mutant *E. coli arg* I gene. Two mutagenic PCR primers were used to synthesize intermediate DNAs of each mutation. Full length mutant *arg* I gene was amplified from two intermediate DNAs by using the 5′- and 3′-terminal primers. Agarose gel electrophoresis was performed to check the correct size of DNA newly systhesized. Amplified mutant *arg* I gene was ligated to the pKK223-3 *E. coli* expression vector, and the construct was transformed to *E. coli* TB2 cell which had a partially deleted *arg* I gene.

Purification of ornithine transcarbamylase : A colony of TB2 cell containing the recombinant plasmid was selected from the M9/uracil/ampicillin/agar plate, and cultured in 2 liter of LB broth with 50μg/ml of ampicillin for overnight at 37℃. The cells were precipitated and suspended in 2 volumes of breakage buffer (100mM KH_2PO_4/40mM ornithine, pH7.5). Cell lysate was heat-denatured at 65℃ for 10 min and cooled down in an ice bath. The resulting supernatant was ammonium sulfate fractionated and dialyzed. The concentrated enzyme solution was applied to the Matrex Blue Gel A column eluting with 0-0.5M KCl gradient in 20mM Tris-acetate buffer. The homogeniety of the enzyme was identified on SDS PAGE.

RESULTS AND DISCUSSION

To clone 1.0 kilobases of *E. coli* mutant *arg* I genes, which were mutated from Serine-55 to glycine, alanine or cysteine, site-directed mutagenesis was applied by using the DNA polymerase chain reaction. Wild type of *arg* I gene cloned in pKK 223-3 expression vector was used as the template. Two mutagenic primers, and 5′- and 3′-end primers were used to amplify two mutagenic intermediate DNAs. The result of gel electrophoresis for 5′- and 3′-side intermediates amplified were shown in Figure 1. The size of 5′-side intermediates was about 0.2 kilobases, and 3′-side intermediates was about 0.8 kilobases.

Full-length mutant *arg* I genes were amplified by using the 5′- and 3′-end primers, and two intermediates as PCR templates.

1 2 3 4 5 6 7

Figure 1. 5′-and 3′-DNA Intermediates made by PCR
1. **5′-Intermediate of SOC 55(Ser→Cys)**
2. **5′-Intermediate of SOG 55(Ser→Gly)**
3. **5′-Intermediate of SOA 55(Ser→Ala)**
4. **Marker(1.35kb, 1.08kb, 0.87kb, 0.60kb, 0.31kb, and 0.2-0.28kb)**
5. **3′-Intermediate of SOC 55(Ser→Cys)**
6. **3′-Intermediate of SOG 55(Ser→Gly)**
7. **3′-Intermediate of SOA 55(Ser→Ala)**

Figure 2 showed the result of gel electrophoresis for mutant *arg* I genes synthesized. Mutant *arg* I genes and expression vector pKK223-3 were double digested with Hind III and EcoR I, and purified from the low melting agarose gel. Each mutant *arg* I gene was ligated to 4.5 kilobases of the pKK223-3, and transformed into *E. coli* XL1 Blue strain. Plasmids were purified by the alkali method, and double digested with Hind III and

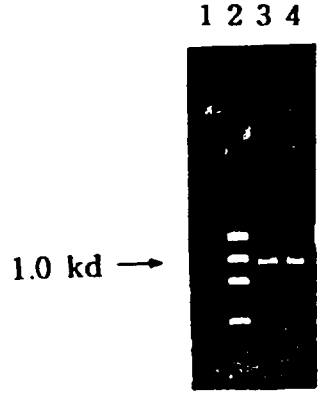

Figure 2. Mutant arg I genes made by PCR.
1. **SOC 55 arg I (Ser→Cys)**
2. **Marker(1.35kb, 1.08kb, 0.87kb, and 0.6kb)**
3. **SOG 55 arg I (Ser→Gly)**
4. **SOA 55 arg I (Ser→Ala)**

EcoR I. Figure 3. showed the result of gel electrophoresis for enzyme digested plamids. The result indicated 1.0 kilobases of mutant *arg* I genes were successfully obtained.

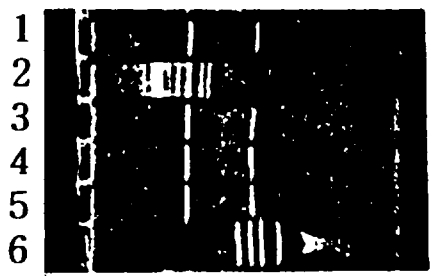

Figure 3. The Result of gel Electrophoresis after HindIII /EcoR I digestion of Mutant arg I /pkk223-3.

1. **HindIII/EcoR I cut of SOC 55 arg I /pkk**
2. **Marker(23.1kb, 9.4kb, 6.6kb, 4.4kb, 2.3kb, 2.0kb and 0.6kb)**
3. **HindIII/EcoR I cut of SOG 55 arg I /pkk**
4. **HindIII/EcoR I cut of SOA 55 arg I /pkk**
5. **HindIII/EcoR I cut of pkk/arg I**
6. **Marker(1.35kb, 1.08kb, 0.87kb, and 0.6kb)**

Purified plasmids were transformed into *E. coli* TB2 strain whose *arg* I gene was partially deleted. Wild type and each mutant ornithine transcarbamylases were expressed from each TB2 cell line in LB/ampicillin medium. The result of SDS-PAGE for purified wild-type enzyme was shown in Figure 4. And the purification profile of the Cysteine-55 mutant enzyme was shown in Figure 5.

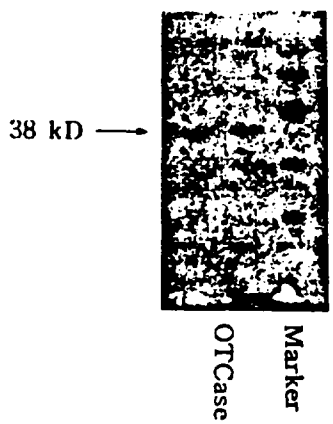

Figure 4. The Result of SDS-PAGE of Wild type OTCase.

The cloning of Ser-55 to Cys, Ala, or Gly mutant *arg* I genes were successfully done. Further works to do are DNA sequencing of mutant *arg* I genes, and more purifications of enzymes, and kinetic characterization of mutant enzymes to elucidate the role of Ser-55 in the catalysis.

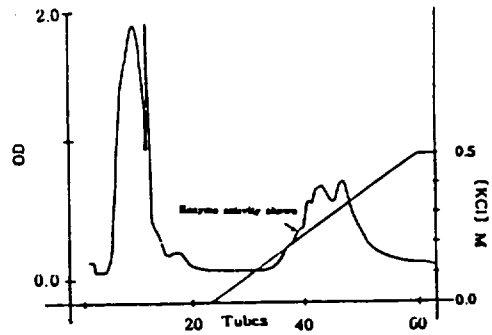

Figure 5. Purification Profile of Cysteine-55 Mutant *E. coli* Ornithine Transcarbamylase

REFERENCES

1. Kantrowitz, E. R. & W. N. Lipscomb (1988) Science **241**, 669-674.
2. Kuo, L. C., C. C. Caron, S. Lee, & W. Herzberg (1990) J. Mol. Biol. **211**, 272-280.
3. Kuo, L. C., A. W. Miller, S. Lee & C. Kozuma (1988) Biochemistry **27**, 8823-8832.